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(54) **COMPOSITIONS COMPRISING  
PHOSPHODIESTERASE INHIBITORS FOR  
THE TREATMENT OF SEXUAL  
DISFUNCTION**

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(52) **U.S. Cl.** ..... **514/250**

(58) **Field of Search** ..... 514/250

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(57) **ABSTRACT**

The present invention relates to highly selective phosphodi-  
esterase (PDE) enzyme inhibitors and to their use in phar-  
maceutical articles of manufacture. In particular, the present  
invention relates to potent inhibitors of cyclic guanosine  
3',5'-monophosphate specific phosphodiesterase type 5  
(PDE5) that when incorporated into a pharmaceutical prod-  
uct at about 1 to about 20 mg unit dosage are useful for the  
treatment of sexual dysfunction.

**12 Claims, No Drawings**

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**COMPOSITIONS COMPRISING  
PHOSPHODIESTERASE INHIBITORS FOR  
THE TREATMENT OF SEXUAL  
DYSFUNCTION**

**CROSS-REFERENCE TO RELATED  
APPLICATIONS**

This is the U.S. national phase application of International Application No. PCT/US00/11129, filed on Apr. 26, 2000, which claims the benefit of provisional patent application Ser. No. 60/132,036, filed Apr. 30, 1999.

**FIELD OF THE INVENTION**

The present invention relates to a highly selective phosphodiesterase (PDE) enzyme inhibitor and to its use in a pharmaceutical unit dosage form. In particular, the present invention relates to a potent inhibitor of cyclic guanosine 3',5'-monophosphate specific phosphodiesterase type 5 (PDE5) that when incorporated into a pharmaceutical product is useful for the treatment of sexual dysfunction. The unit dosage form described herein is characterized by selective PDE5 inhibition, and accordingly, provides a benefit in therapeutic areas where inhibition of PDE5 is desired, with minimization or elimination of adverse side effects resulting from inhibition of other phosphodiesterase enzymes.

**BACKGROUND OF THE INVENTION**

The biochemical, physiological, and clinical effects of cyclic guanosine 3',5'-monophosphate specific phosphodiesterase (cGMP-specific PDE) inhibitors suggest their utility in a variety of disease states in which modulation of smooth muscle, renal, hemostatic, inflammatory, and/or endocrine function is desired. Type 5 cGMP-specific phosphodiesterase (PDE5) is the major cGMP hydrolyzing enzyme in vascular smooth muscle, and its expression in penile corpus cavernosum has been reported (Taher et al., *J. Urol.*, 149, p. 285A (1993)). Thus, PDES is an attractive target in the treatment of sexual dysfunction (Murray, *DN&P* 6(3), pp. 150-56 (1993)).

A pharmaceutical product, which provides a PDE5 inhibitor, is currently available and marketed under the trademark VIAGRA®. The active ingredient in VIAGRA® is sildenafil. The product is sold as an article of manufacture including 25, 50, and 100 mg tablets of sildenafil and a package insert. The package insert provides that sildenafil is a more potent inhibitor of PDE5 than other known phosphodiesterases (greater than 80 fold for PDE1 inhibition, greater than 1,000 fold for PDE2, PDE3, and PDE4 inhibition). The IC<sub>50</sub> for sildenafil against PDE5 has been reported as 3 nM (*Drugs of the Future*, 22(2), pp. 138-143 (1997)) and as 3.9 nM (Boolel et al., *Int. J. of Impotence*, 8, pp. 47-52 (1996)). Sildenafil is described as having a 4,000-fold selectivity for PDE5 versus PDE3, and only a 10-fold selectivity for PDE5 versus PDE6. Its relative lack of selectivity for PDE6 is theorized to be the basis for abnormalities related to color vision.

While sildenafil has obtained significant commercial success, it has fallen short due to its significant adverse side effects, including facial flushing (10% incidence rate). Adverse side effects limit the use of sildenafil in patients suffering from vision abnormalities, hypertension, and, most significantly, by individuals who use organic nitrates (Welds et al., *Amer. J. of Cardiology*, 83(5A), pp. 21(C)-28(C) (1999)).

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could place the patient in danger. Accordingly, the package label for sildenafil provides strict contraindications against its use in combination with organic nitrates (e.g., nitroglycerin, isosorbide mononitrate, isosorbide nitrate, erythryl tetranitrate) and other nitric oxide donors in any form, either regularly or intermittently, because sildenafil potentiates the hypotensive effects of nitrates. See C. R. Conti et al., *Amer. J. of Cardiology*, 83(5A), pp. 29C-34C (1999). Thus, even with the availability of sildenafil, there remains a need to identify improved pharmaceutical products that are useful in treating sexual dysfunction.

Daugan U.S. Pat. No. 5,859,006 discloses certain tetracyclic derivatives that are potent inhibitors of cGMP-specific PDE, or PDES. The IC<sub>50</sub> of the compounds disclosed in U.S. Pat. No. 5,859,006 is reported in the range of 1 nM to 10 μM. The oral dosage for such compounds is 0.58 mg daily for an average adult patient (70 kg). Thus, unit dosage forms (tablets or capsules) are reported as 0.2 to 400 mg of active compound. Significant adverse side effects attributed to compounds disclosed in U.S. Pat. No. 5,859,006 are not disclosed.

Applicants have discovered that one such tetracyclic derivative, (6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione, alternatively named (6R-trans)-6-(1,3-benzodioxol-5-yl)-2,3,6,7,12,12a-hexahydro-2-methylpyrazino-[1',2':1,6]pyrido[3,4-b]indole-1,4-dione, and referred to herein as Compound (I), can be administered in a unit dose that provides an effective treatment without the side effects associated with the presently marketed PDE5 inhibitor, sildenafil. Prior to the present invention such side effects were considered inherent to the inhibition of PDE5.

Significantly, applicants' clinical studies also reveal that an effective product having a reduced tendency to cause flushing in susceptible individuals can be provided. Most unexpectedly, the in product also can be administered with clinically insignificant side effects associated with the combined effects of a PDE5 inhibitor and an organic nitrate. Thus, the contraindication once believed necessary for a product containing a PDE5 inhibitor is unnecessary when Compound (I) is administered as a unit dose of about 1 to about 20 mg, as disclosed herein. Thus, the present invention provides an effective therapy for sexual dysfunction in individuals who previously were untreatable or suffered from unacceptable side effects, including individuals having cardiovascular disease, such as in individuals requiring nitrate therapy, having suffered a myocardial infarction more than three months before the onset of sexual dysfunction therapy, and suffering from class 1 congestive heart failure, or individuals suffering from vision abnormalities.

The present invention provides Compound (I) in a unit dosage form. That is, the present invention provides a pharmaceutical unit dosage form suitable for oral administration comprising about 1 to about 20 mg Compound (I).

**SUMMARY OF THE INVENTION**

The present invention provides a pharmaceutical dosage form for human pharmaceutical use, comprising about 1 to about 20 mg of (6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione in a unit dosage form suitable for oral administration.

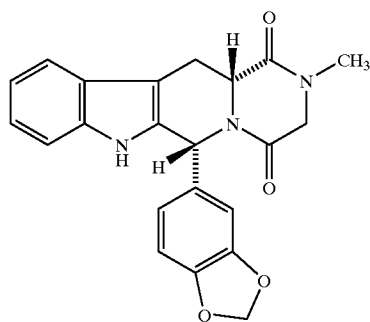
The present invention further provides a method of treating conditions where inhibition of PDE5 is desired, which

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PDE5 inhibitor, as needed, up to a total dose of 20 mg per day. The invention further provides the use of an oral dosage form comprising a selective PDE5 inhibitor at a dosage of about 1 to about 20 mg for the treatment of sexual dysfunction.

Specific conditions that can be treated by the present invention, include, but are not limited to, male erectile dysfunction and female sexual dysfunction, particularly female arousal disorder, also known as female sexual arousal disorder.

In particular, the present invention is directed to a pharmaceutical unit dosage composition comprising about 1 to about 20 mg of a compound having the structural formula:



said unit dosage form suitable for oral administration, and method of treating sexual dysfunction using the pharmaceutical unit dose composition.

#### DETAILED DESCRIPTION

For purposes of the present invention as disclosed and described herein, the following terms and abbreviations are defined as follows.

The term “container” means any receptacle and closure therefor suitable for storing, shipping, dispensing, and/or handling a pharmaceutical product.

The term “ $IC_{50}$ ” is the measure of potency of a compound to inhibit a particular PDE enzyme (e.g., PDE1c, PDE5, or PDE6). The  $IC_{50}$  is the concentration of a compound that results in 50% enzyme inhibition in a single dose-response experiment. Determining the  $IC_{50}$  value for a compound is readily carried out by a known in vitro methodology generally described in Y. Cheng et al., *Biochem. Pharmacol.*, 22, pp. 3099–3108 (1973).

The term “package insert” means information accompanying the product that provides a description of how to administer the product, along with the safety and efficacy data required to allow the physician, pharmacist, and patient to make an informed decision regarding use of the product. The package insert generally is regarded as the “label” for a pharmaceutical product.

The term “oral dosage form” is used in a general sense to reference pharmaceutical products administered orally. Oral dosage forms are recognized by those skilled in the art to include such forms as liquid formulations, tablets, capsules, and gencaps.

The term “vision abnormalities” means abnormal vision characterized by blue-green vision believed to be caused by PDE6 inhibition.

The term “flushing” means an episodic redness of the face and neck attributed to vasodilation caused by ingestion of a

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The term “free drug” means solid particles of drug not intimately embedded in a polymeric coprecipitate.

The presently claimed dosage form preferably is packaged as an article of manufacture for human pharmaceutical use, comprising a package insert, a container, and a dosage form comprising about 1 to about 20 mg of Compound (I)

The package insert provides a description of how to administer a pharmaceutical product, along with the safety and efficacy data required to allow the physician, pharmacist, and patient to make an informed decision regarding the use of the product. The package insert generally is regarded as the label of the pharmaceutical product. The package insert incorporated into the article of manufacture indicates that Compound (I) is useful in the treatment of conditions wherein inhibition of PDE5 is desired. The package insert also provides instructions to administer one or more about 1 to about 20 mg unit dosage forms as needed, up to a maximum total dose of 20 mg per day. Preferably, the dose administered is about 5 to about 20 mg/day, more preferably about 5 to about 15 mg/day. Most preferably, a 10 mg dosage form is administered once per day.

Preferred conditions to be treated include sexual dysfunction (including male erectile dysfunction; and female sexual dysfunction, and more preferably female arousal disorder (FAD)). The preferred condition to be treated is male erectile dysfunction.

Significantly, the package insert supports the use of the product to treat sexual dysfunction in patients suffering from a retinal disease, for example, diabetic retinopathy or retinitis pigmentosa, or in patients who are using organic nitrates. Thus, the package insert preferably is free of contraindications associated with these conditions, and particularly the administration of the dosage form with an organic nitrate. More preferably, the package insert also is free of any cautions or warnings both associated with retinal diseases, particularly retinitis pigmentosa, and associated with individuals prone to vision abnormalities. Preferably, the package insert also reports incidences of flushing below 2%, preferably below 1%, and most preferably below 0.5%, of the patients administered the dosage form. The incidence rate of flushing demonstrates marked improvement over prior pharmaceutical products containing a PDE5 inhibitor.

The container used in the article of manufacture is conventional in the pharmaceutical arts. Generally, the container is a blister pack, foil packet, glass or plastic bottle and accompanying cap or closure, or other such article suitable for use by the patient or pharmacist. Preferably, the container is sized to accommodate 1–1000 solid dosage forms, preferably 1 to 500 solid dosage forms, and most preferably, 5 to 30 solid dosage forms.

Oral dosage forms are recognized by those skilled in the art to include, for example, such forms as liquid formulations, tablets, capsules, and gencaps. Preferably the dosage forms are solid dosage forms, particularly, tablets comprising about 1 to about 20 mg of Compound (I). Any pharmaceutically acceptable excipients for oral use are suitable for preparation of such dosage forms. Suitable pharmaceutical dosage forms include coprecipitate forms described, for example, in Butler U.S. Pat. No. 5,985,326, incorporated herein by reference. In preferred embodiments, the unit dosage form of the present invention is a solid free of a coprecipitate form of Compound (I), but rather contains solid Compound (I) as a free drug.

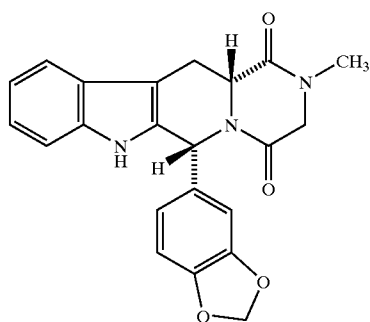
Preferably, the tablets comprise pharmaceutical excipi-

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stearate, stearic acid, talc, and colloidal silicon dioxide, and are prepared by standard pharmaceutical manufacturing techniques as described in *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, Pa. (1990). Such techniques include, for example, wet granulation followed by drying, milling, and compression into tablets with or without film coating; dry granulation followed by milling, compression into tablets with or without film coating; dry blending followed by compression into tablets, with or without film coating; molded tablets; wet granulation, dried and filled into gelatin capsules; dry blend filled into gelatin capsules; or suspension and solution filled into gelatin capsules. Generally, the solid dosage forms have identifying marks which are debossed or imprinted on the surface.

The present invention is based on detailed experiments and clinical trials, and the unexpected observations that side effects previously believed to be indicative of PDE5 inhibition can be reduced to clinically insignificant levels by the selection of a compound and unit dose. This unexpected observation enabled the development of a unit dosage form that incorporates Compound (I) in about 1 to about 20 mg per unit dosage forms that, when orally administered, minimizes undesirable side effects previously believed unavoidable. These side effects include facial flushing, vision abnormalities, and a significant decrease in blood pressure, when Compound (I) is administered alone or in combination with an organic nitrate. The minimal effect of Compound (I), administered in about 1 to about 20 mg unit dosage forms, on PDE6 also allows the administration of a selective PDE5 inhibitor to patients suffering from a retinal disease, like diabetic retinopathy or retinitis pigmentosa.

Compound (I) has the following structural formula:



The compound of structural formula (I) was demonstrated in human clinical studies to exert a minimal impact on systolic blood pressure when administered in conjunction with organic nitrates. By contrast, sildenafil demonstrates a four-fold greater decrease in systolic blood pressure over a placebo, which leads to the contraindications in the VIA-GRA insert, and in warnings to certain patients.

The following illustrates the PDE5 and PDE6 IC<sub>50</sub> values for the compound of structural formula (I) determined by the procedures described herein.

Compound	PDE5 IC <sub>50</sub> (nM)	PDE6 IC <sub>50</sub> (nM)	PDE6/PDE5
I	2.5	3400	1360

The compound of structural formula (I) additionally dem-

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## PREPARATIONS

## Human PDE5 Preparation

Recombinant production of human PDE5 was carried out essentially as described in Example 7 of U.S. Pat. No. 5,702,936, incorporated herein by reference, except that the yeast transformation vector employed, which is derived from the basic ADH2 plasmid described in V. Price et al., *Methods in Enzymology*, 1985, pages 308-318 (1990), incorporated yeast ADH2 promoter and terminator sequences rather than ADH1 promoter and terminator sequences and the *Saccharomyces cerevisiae* host was the protease-deficient strain BJ2-54 deposited on Aug. 31, 1998 with the American Type Culture Collection, Manassas, Va., under accession number ATCC 74465. Transformed host cells were grown in 2xSC-leu medium, pH 6.2, with trace metals, and vitamins. After 24 hours, YEP medium containing glycerol was added to a final concentration of 2xYEP/3% glycerol. Approximately 24 hours later, cells were harvested, washed, and stored at -700C.

Cell pellets (29 g) were thawed on ice with an equal volume of lysis buffer (25 mM Tris-Cl, pH 8, 5 mM MgCl<sub>2</sub>, 0.25 mM dithiothreitol, 1 mM benzamide, and 10 μM ZnSO<sub>4</sub>). Cells were lysed in a microfluidizer with N<sub>2</sub> at 20,000 psi. The lysate was centrifuged and filtered through 0.45 μm disposable filters. The filtrate was applied to a 150 mL column of Q Sepharose Fast Flow (Pharmacia). The column was washed with 1.5 volumes of Buffer A (20 mM Bis-Tris Propane, pH 6.8, 1 mM MgCl<sub>2</sub>, 0.25 mM dithiothreitol, 10 μM ZnSO<sub>4</sub>) and eluted with a step gradient of 125 AM NaCl in Buffer A followed by a linear gradient of 125-1000 mM NaCl in Buffer A.

Active fractions from the linear gradient were applied to a 180 mL ceramic hydroxyapatite column in Buffer B (20 mM Bis-Tris Propane (pH 6.8), 1 MM MgCl<sub>2</sub>, 0.25 mM dithiothreitol, 10 μM ZnSO<sub>4</sub>, and 250 mM KCl). After loading, the column was washed with 2 volumes of Buffer B and eluted with a linear gradient of 0-125 mM potassium phosphate in Buffer B. Active fractions were pooled, precipitated with 60% ammonium-sulfate, and resuspended in Buffer C (20 mM Bis-Tris Propane, pH 6.8, 125 mM NaCl, 0.5 mM dithiothreitol, and 10 μM ZnSO<sub>4</sub>). The pool was applied to a 140 mL column of Sephacryl S-300 HR and eluted with Buffer C. Active fractions were diluted to 50% glycerol and stored at -20° C. The resultant preparations were about 85% pure by SDS-PAGE.

## Assay for PDE Activity

Activity of PDE5 can be measured by standard assays in the art. For example, specific activity of any PDE can be determined as follows. PDE assays utilizing a charcoal separation technique were performed essentially as described in Loughney et al., (1996), *The Journal of Biological Chemistry*, 271:796-806. In this assay, PDE5 activity converts [<sup>32</sup>p]cGMP to [32p]5'GMP in proportion to the amount of PDE5 activity present. The [32P]5'GMP then is quantitatively converted to free [32P] phosphate and unlabeled adenosine by the action of snake venom 5'-nucleotidase. Hence, the amount of [32P] phosphate liberated is proportional to enzyme activity. The assay is performed at 30 C in a 100 μL reaction mixture containing (final concentrations) 40 mM Tris-Cl (pH 8.0), 1 μM ZnSO<sub>4</sub>, 5 mM MgCl, and 0.1 mg/mL bovine serum albumin. PDE5 is present in quantities that yield <30% total hydrolysis of substrate (linear assay conditions). The assay is initiated by addition of substrate (1 nM [32P]cGMP), and the mixture is incubated for 12 minutes. Seventy-five (75) μg of *Crotalus*

stopped by addition of 200 mL of activated charcoal (25 mg/mL suspension in 0.1 M  $\text{NaH}_2\text{PO}_4$ , pH 4). After centrifugation (750  $\times$ g for 3 minutes) to sediment the charcoal, a sample of the supernatant is taken for radioactivity determination in a scintillation counter and the PDE5 activity is calculated. The preparations had specific activities of about 3  $\mu$ moles cGMP hydrolyzed per minute per milligram protein.

#### Bovine PDE6 Preparation

Bovine PDE6 was supplied by Dr. N. Virmaux, INSERM U338, Strasbourg. Bovine retinas were prepared as described by Virmaux et al., *FEBS Letters*, 12(6), pp. 325–328 (1971) and see also, A. Sitaramayya et al., *Exp. Eye Res.*, 25, pp. 163–169 (1977). Briefly, unless stated otherwise, all operations were done in the cold and in dim red light. Eyes were kept in the cold and in the dark for up to four hours after slaughtering.

Preparation of bovine retinal outer segment (ROS) basically followed procedures described by Schichi et al., *J. Biol. Chem.*, 224:529 (1969). In a typical experiment, 35 bovine retinas were ground in a mortar with 35 mL 0.066 M phosphate buffer, pH 7.0, made up to 40% with sucrose, followed by homogenization in a Potter homogenizer (20 up and down strokes). The suspension was centrifuged at 25,000 $\times$ g for 20 minutes. The pellet was homogenized in 7.5 mL 0.066 M phosphate buffer (40% in sucrose), and carefully layered under 7.5 mL of phosphate buffer (containing no sucrose). Centrifugation was conducted in a swing-out rotor at 45,000 $\times$ g for 20 minutes, and produced a pellet which is black at the bottom, and also a red band at the interface 0.066 M phosphate—40% sucrose/0.066 M phosphate (crude ROS). The red material at the interface was removed, diluted with phosphate buffer, spun down to a pellet, and redistributed in buffered 40% sucrose as described above. This procedure was repeated 2 or 3 times until no pellet was formed. The purified ROS was washed in phosphate buffer and finally spun down to a pellet at 25,000 $\times$ g for 20 minutes. All materials were then kept frozen until used.

Hypotonic extracts were prepared by suspending isolated ROS in 10 mM Tris-Cl pH 7.5, 1 mM EDTA, and 1 mM dithioerythritol, followed by centrifugation at 100,000 $\times$ g for 30 minutes.

The preparation was reported to have a specific activity of about 35 nmoles cGMP hydrolyzed per minute per milligram protein.

#### PDE1c Preparation from *Spodoptera fugiperda* Cells (Sf9)

Cell pellets (5g) were thawed on ice with 20 ml of Lysis Buffer (50 mM MOPS pH 7.4, 10  $\mu$ M  $\text{ZnSO}_4$ , 0.1 mM  $\text{CaCl}_2$ , 1 mM DTT, 2mM benzamidine HCl, 5  $\mu$ g/ml each of pepstatin, leupeptin, and aprotinin). Cells were lysed by passage through a French pressure cell (SLM-Aminco) while temperatures were maintained below 10° C. The resultant cell homogenate was centrifuged at 36,000 rpm at 4° C. for 45 minutes in a Beckman ultracentrifuge using a Type TI45 rotor. The supernatant was discarded and the resultant pellet was resuspended with 40 ml of Solubilization Buffer (Lysis Buffer containing 1M NaCl, 0.1M  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 20  $\mu$ g/ml calmodulin, and 1% Sulfobetaine SB12 (Z3-12) by sonicating using a VibraCell tuner with a microtip for 3 $\times$ 30 seconds. This was performed in a crushed ice/salt mix for cooling. Following sonication, the mixture was slowly mixed for 30 minutes at 4° C. to finish solubilizing membrane bound proteins. This mixture was centrifuged in a Beckman ultracentrifuge using a type TI45 rotor

II. The precipitated protein was centrifuged for 20 minutes at 9,000 rpm in a Beckman JA-10 rotor. The recovered supernatant then was subjected to Mimetic Blue AP Agarose Chromatography.

In order to run the Mimetic Blue AP Agarose Column, the resin initially was shielded by the application of 10 bed volumes of 1% polyvinylpyrrolidone (i.e., MW of 40,000) to block nonspecific binding sites. The loosely bound PVP-40 was removed by washing with 10 bed volumes of 2M NaCl, and 10 mM sodium citrate pH 3.4. Just prior to addition of the solubilized PDE1c3 sample, the column was equilibrated with 5 bed volumes of Column Buffer A (50 mM MOPS pH 7.4, 10  $\mu$ M  $\text{ZnSO}_4$ , 5mM  $\text{MgCl}_2$ , 0.1 mM  $\text{CaCl}_2$ , 1 mM DTT, 2 mM benzamidine HCl).

The solubilized sample was applied to the column at a flow rate of 2 ml/min with recycling such that the total sample was applied 4 to 5 times in 12 hours. After loading was completed, the column was washed with 10 column volumes of Column Buffer A, followed by 5 column volumes of Column Buffer B (Column Buffer A containing 20 mM 5'-AMP), and followed by 5 column volumes of Column Buffer C (50 mM MOPS pH 7.4, 10  $\mu$ M  $\text{ZnSO}_4$ , 0.1 mM  $\text{CaCl}_2$ , 1 mM dithiothreitol, and 2 mM benzamidine HCl). The enzyme was eluted into three successive pools. The first pool consisted of enzyme from a 5 bed volume wash with Column Buffer C containing 1 mM cAMP. The second pool consisted of enzyme from a 10 bed volume wash with Column Buffer C containing 1 M NaCl. The final pool of enzyme consisted of a 5 bed volume wash with Column Buffer C containing 1 M NaCl and 20 mM cAMP.

The active pools of enzyme were collected and the cyclic nucleotide removed via conventional gel filtration chromatography or chromatography on hydroxy-apatite resins. Following removal of cyclic nucleotides, the enzyme pools were dialyzed against Dialysis Buffer containing 25 mM MOPS pH 7.4, 10  $\mu$ M  $\text{ZnSO}_4$ , 500 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM dithiothreitol, 1 mM benzamidine HCl, followed by dialysis against Dialysis buffer containing 50% glycerol. The enzyme was quick frozen with the aid of dry ice and stored at -70° C.

The resultant preparations were about >90% pure by SDS-PAGE. These preparations had specific activities of about 0.1 to 1.0  $\mu$ mol cAMP hydrolyzed per minute per milligram protein.

#### IC<sub>50</sub> Determinations

The parameter of interest in evaluating the potency of a competitive enzyme inhibitor of PDE5 and/or PDE1c and PDE6 is the inhibition constant, i.e.,  $K_i$ . This parameter can be approximated by determining the ICS<sub>50</sub>, which is the inhibitor concentration that results in 50% enzyme inhibition, in a single dose-response experiment under the following conditions.

The concentration of inhibitor is always much greater than the concentration of enzyme, so that free inhibitor concentration (which is unknown) is approximated by total inhibitor concentration (which is known).

A suitable range of inhibitor concentrations is chosen (i.e., inhibitor concentrations at least several fold greater and several fold less than the  $K_i$  are present in the experiment). Typically, inhibitor concentrations ranged from 10 nM to 10  $\mu$ M.

The concentrations of enzyme and substrate are chosen such that less than 20% of the substrate is consumed in the absence of inhibitor (providing, e.g., maximum substrate hydrolysis of from 10 to 15%), so that enzyme activity is approximately constant throughout the assay.

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